A1 Adenosine Receptors of Bovine Brain Couple to Guanine Nucleotide-binding Proteins G11, G12, and G0*

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Many receptors regulate various effector systems through a family of guanine nucleotide-binding regulatory proteins (G proteins). There are more than 10 members in the G protein family each of which is a heterotrimer composed of α, β, and γ subunits (7, 8). A1 receptors have been shown to act via several different effectors. These include inhibition of adenylate cyclase (1), activation of potassium channels (9, 10), inactivation of Ca2+ channels (11), and enhancement (12) or inhibition of phospholipase C (13). All of these A1 receptor-mediated responses are sensitive to inhibition by Bordetella pertussis toxin (14, 15) and N-ethylmaleimide (NEM; 2, 15, 16), agents that uncouple the inhibitory G proteins G9 and G12. It is therefore thought that A1 receptors may couple predominantly to pertussis toxin-sensitive G proteins (15).

We have co-purified A1 receptors and associated G proteins of bovine brain by affinity chromatography on an agonist affinity column (17). Pertussis toxin catalyzes the ADP-ribosylation of at least two distinct polypeptides (39- and 41-kDa molecular mass) in the purified fraction, suggesting that solubilized A1 receptors are complexed with G9 and one or more of the G9 family of G proteins. Some G protein βγ subunits are present in affinity column eluates since ADP-ribosylation of these fractions does not require exogenous βγ (17). Also, purified bovine brain A1 receptors couple selectively to pertussis toxin-sensitive G proteins of human platelet membranes after reconstitution (15). In this study we have used specific antipeptide antisera to show that A1 receptors co-elute from the affinity column primarily with G9, and with much less G12. We also demonstrate that these three G proteins purified from bovine brain can be functionally reconstituted in phospholipid vesicles with purified A1 receptors.

EXPERIMENTAL PROCEDURES

Chemicals—Carrier-free Na[35S] (2,200 Ci/mmol), [35S]GTPγS (1,180–1,240 Ci/mmol), and [α-32P]GTP (3,000 Ci/mmol) were purchased from New England Nuclear. CHAPS, NEM, HEPES, Tris, GTP, dichloroacetate, adenosine, adenosine deaminase, phenylmethylsulfonyl fluoride, benzamidine, and sodium chloride were from Sigma. R-PJA and GTPγS were from Boehringer Mannheim; ascleptin, from Associated Concentrates, Long Island, NY; BA85 nitrocellulose membrane filters, from Schleicher & Schuell; polyvinylidene difluoride transfer membranes, (Immobilon-P) from Millipore Inc. Bedford, MA and PEG-6 desalting resin, from Bio-Rad. The syntheses and purification of ABA, 125I-ABA, and 125I-BW-A844U86 were as described previously (2, 4, 5, 17).

1 The abbreviations used are: G proteins, guanine nucleotide-binding regulatory proteins; HEPES, N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate; NEM, N-ethylmaleimide; 125I-ABA, [125I]N(3-iodo-4-amino)benzyladenosine; 125I-BW-A844U86, [125I]3-(4-aminomethyl)iodo]phenethyl-8-cyclopentylxanthine; R-PJA, R-phenylisopropyladenosine; GTPγS, guanosine 5’-O-(3-thio)triphosphate; SDS, sodium dodecyl sulfate.
Preparation of A1 Adenosine Receptors and G Proteins—Bovine cerebral cortical membranes were solubilized with the detergent CHAPS and A1 receptors purified by affinity chromatography over aminobenzyladenosine (ABA)-Affi-Gel 202 affinity columns essentially as described (17). The purified receptors in buffer A (10 mM HEPES, 1 mM EDTA, 0.1 mM benzamidine) containing 10% glycerol, 0.1 M NaCl, 0.1% CHAPS, and 0.01% asolectin and either 10 μM GTP or 200 μM NEM were stored frozen at −20 °C. The Bmax for binding of [35S]GTPγS to purified receptors used for reconstitution ranged between 83 and 141 pmol/mg protein (184-313-fold enriched over crude soluble receptors). G protein subunits (Gα1, Gα2, Gβ1, and Gγ) were isolated from bovine brain membranes as described (18-20).

Identification of G Protein Subunits by Immunoblotting—G proteins subunits were identified by Western blotting using antipeptide antisera. A total of 1.4 nmol of [35S]GTPγS binding sites was co-purified with A1 adenosine receptors from 500 g (wet weight) of bovine brain. The G proteins were concentrated to 200 μl by Amicon filtration (YM-10 membrane). Various amounts (2.25-13.5 μl) were loaded per lane of polyacrylamide gels. After electrophoresis, the peptides were transblotted at 4 °C on Immobilon-P (polyvinylidene difluoride type) transfer membranes at 100 V for 1 h (21). Prestained standards (Bio-Rad) were used to monitor protein transfer. The blots were incubated with antisera, and, after washing, immune complexes were detected with 125I-labeled goat antirabbit F(ab′)2 fragments (Molecular Probes). G protein subunits were visualized by autoradiography, using Kodak X-1 film in Du Pont x-ray cassettes with Cronex intensifying screens at −70 °C for 4 days. Goat anti-rabbit F(ab′)2 fragments (ImmunoResearch) were iodinated using carrier-free 125I and Enzymobeads (Bio-Rad).

Reconstitution of Receptors and G Proteins into Phospholipid Vesicles—Receptors used for reconstitution with purified G proteins were eluted from the affinity column with 100 μM NEM to inactivate completely the endogenous G protein α subunits that co-elute with receptors. Receptors were treated with 2 mM dithiothreitol to quench residual NEM (15, 16) and then incubated with 20 μM adenosine at 21 °C for 20 min. Reconstitution was performed essentially as described for muscarinic receptors (22). Typically, the reconstitution mixture contained 0.45-1.7 pmol of A1 receptors ([35S]GTPγS binding sites), 10 μM adenosine, 0.2% asolectin (95% soybean phosphatides), 0.05% CHAPS, 10 mM MgCl2, 5% glycerol, 0.1 M NaCl, 2 mM dithiothreitol, and various amounts of G protein α and βγ subunits in a final volume of 200 μl. After incubation on ice for 1-1.5 h, incorporation of receptors and G proteins into asolectin vesicles was achieved by detergent removal using P6DG desalting columns. Reconstitution was assessed by measuring high affinity [35S]ABA binding to receptor and R-PIA-stimulated [35S]GTPγS binding to G proteins after 40-fold dilution of the vesicles as described below. The concentrations of purified α and βγ subunits added was based on determinations of [35S]GTPγS binding and protein, respectively.

Radioligand Binding Assays—Receptor binding assays were performed essentially as described (5, 17). The bound and free radioligand was separated by filtration on Whatman GF/B glass fiber filters pretreated with 0.3% polyethyleneimine (23). [35S]GTPγS binding was determined as described previously (17). Low molecular mass GTP-binding proteins were assayed by autoradiography after electrophoresis, transblotted onto polyvinylidene difluoride membranes, and incubation of the membranes with [α-32P]GTP in the presence and absence of 100 μM GTP (24).

RESULTS

A1 adenosine receptors and associated G proteins were eluted from ABA-Affi-Gel 202 in the presence of GTP or NEM and quantitated based on the binding of 125I-IBW-A844U86 and [35S]GTPγS, respectively. No competing receptor ligands were required to facilitate elution from the affinity column because of the drop in the affinity of receptors for agonists elicited by GTP or NEM (5). Low molecular mass GTP-binding proteins could not be detected, although such G proteins were detected in crude brain membranes and their detergent extracts (data not shown).

Specific antisera raised against peptides representing sequences from Gα1, Gα2, and Gα3 were used to detect α subunits coupled to A1 adenosine receptors. Immunoblotting to standard G protein subunits purified from bovine brain and G protein subunits co-purified by affinity chromatography with A1 adenosine receptors are shown in lanes 1 and 2, respectively of Fig. 1. By comparing the intensity of the autoradiographs of standards and samples we could conclude that the α subunit binding sites consisted of >37.5% each of Gα1 and Gα2 (Table I). By contrast, relatively little Gα2 was detected (<12.5%). Antisera against Gα3 subunits revealed that both Gα3 and Gα5 polyepptides co-elute with A1 receptors (Fig. 1, D and E). We did not determine if G protein β subunits were purified in amounts stoichiometric with α subunits, but of the β subunits that were eluted from the affinity column, there was a large excess of β3 over β2 (Table I).

Evidence of functional interactions of A1 receptors with Gα1, Gα2, and Gα3 was derived from studies of reconstitution of purified A1 receptors into phospholipid vesicles with G proteins isolated from bovine brain. Fig. 2 shows that excess G protein over receptors is required for optimal reconstitution.

![Fig. 1. Immunological identification of G protein subunits in affinity-purified adenosine receptor-G protein complexes. Homogeneous bovine brain G proteins subunits (lanes 1) and ABA-Affi-Gel 202 column eluates containing 2.4 nmol of [35S]GTPγS binding sites/mg protein (lanes 2) were resolved by SDS-polyacrylamide gel electrophoresis, transblotted onto polyvinylidene difluoride membrane filters, and analyzed by Western blotting using anti-peptide antisera. Because of differences in the affinity of the antisera, variable amounts of standard (G protein subunits) and samples were applied to various lanes. These are tabulated in Table I. A, Gα1, was probed with I-555 antisemur (1:200). B, Gα2 was probed with J-883 (1:1,000). C, Gα3 was probed with U-46 (1:1,000). D, Gα5 was probed with J-887 (1:200). E, Gγ was probed with U-49 (1:2,000). Only the portions of the blots corresponding to G protein subunits are shown; no other bands were detected.

<table>
<thead>
<tr>
<th>Table I</th>
<th>Quantitation of G protein subunits by Western blotting</th>
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<tr>
<td>G protein subunit</td>
<td>Total G protein subunits in sample lane</td>
</tr>
<tr>
<td>Gα1</td>
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<tr>
<td>Gα2</td>
<td>96 pmol</td>
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<td>Gγ</td>
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Fig. 2. Reconstitution of $A_1$ adenosine receptors and G proteins. Receptors (1.66 pmol) were incubated with 6.25–100 pmol of various G proteins (equimolar purified bovine brain α and β subunits) as shown. The proteins were reconstituted into asolectin vesicles as described under "Experimental Procedures." Reconstitution was monitored by measuring $[^{125}]$-ABA binding in the absence and presence of 10 μM GTPyS. Pooled data from two experiments each in triplicate were fit to the equation: $B = B_{max} \times G/ED_{50} + G$, where $B$ is $[^{125}]$-ABA bound and $G$ is the amount of G protein added (expressed as fold excess over the amount of receptors used). Amounts of G proteins needed for reconstitution to half-maximum agonist binding ($ED_{50}$) are: $34 \pm 7$, $4.7 \pm 1.2$, and $24 \pm 5$-fold in excess of the amount of receptors for $G_{α}, G_{β},$ and $G_{γ}$, respectively.

of GTPyS-sensitive high affinity binding of $^{[125]}$-ABA to receptors. A $34$- and $24$-fold excess of $G_{α}$ and $G_{δ}$, respectively, over receptors was required to restore half-maximally the high affinity agonist binding. $G_{α}$ ($ED_{50} = 4.7$-fold) was more potent than $G_{α}$ and $G_{δ}$. Based on the number of receptors used for reconstitution (100 pmol, 10 fmol/0.1 ml), the concentration of $^{[125]}$-ABA (53 or 95 pm) and the $K_D$ of the radioligand (300 pm) we calculated that approximately $25 \pm 5.6\%, n = 2$, of the total receptors are converted to the high affinity state when reconstituted with a large excess of G proteins. The fraction of receptors reconstituted was determined in separate experiments by measuring $^{[125]}$-BW-AB44U86 binding sites in vesicles pelleted by ultracentrifugation and amounted to $28$–$40\%, n = 3$. Therefore, the fraction of receptors reconstituted into vesicles which converts to the high affinity state upon the addition of G proteins may be as high as $60\%$.

Agonist binding to receptor-G protein complexes is thought to stimulate guanine nucleotide exchange by accelerating the dissociation of bound GDP (22). Fig. 3 shows that $R$-PIA accelerates the binding of $[^{35}]$GTPyS to various G proteins reconstituted with $A_1$ receptors into phospholipid vesicles. The rate constants, $k$, for $[^{35}]$GTPyS binding increased 6.5-fold ($G_{α}$ and $G_{α}$) and 8.5-fold ($G_{δ}$) in the presence of $20 \mu$M receptor agonist $R$-PIA (Fig. 3). The binding of $[^{35}]$GTPyS at equilibrium in all cases was $7,400$ cpm (247 pm; Fig. 3). Based on the results shown in Fig. 2 we calculated that 15–25$\%$ of the total receptors added (220 pm) were converted to the high affinity conformation by the 20-fold excess of various G proteins used in this experiment. Therefore we calculated that 33–55 pm receptors were coupled to G proteins. These data suggest that under these assay conditions each activated receptor acted catalytically to release GDP from four to seven G proteins.

**DISCUSSION**

We have reported previously that $A_1$ receptor-G protein complexes can be adsorbed to an agonist affinity column and eluted after washing with NEM or GTP. We have now extended these studies to determine which pertussis toxin-sensitive G proteins in bovine brain can couple to $A_1$ adenosine receptors. The results indicate that $A_1$ adenosine receptors of bovine brain can couple rather indiscriminately to at least three different pertussis toxin-sensitive G proteins (Figs. 2 and 3). These are $G_{α}, G_{α},$ and $G_{α}$. $G_{α}$ was not examined in this study. Immunoblotting revealed that both $β_{α}$ and $β_{δ}$ subunits interact with $A_1$ receptor-G, complexes (Fig. 1, D and E). The amounts of $G_{α}$, $G_{δ}$, and $G_{α}$ found coupled to bovine $A_1$ receptors are at least three times greater than $G_{β_{α}}$ (Fig. 1 B). Since $A_1$ receptors can be reconstituted with $G_{β_{α}}$, more readily than $G_{α}$, and $G_{α}$, the data suggest that $G_{β_{α}}$ may be a relatively minor G protein in bovine brain tissue rich in $A_1$ adenosine receptors. Previous studies suggest that $G_{β_{α}}$ is by far the most abundant and $G_{β_{α}}$ the least abundant G protein in bovine brain (26). $G_{β_{α}}$ was found to be 5- and 16-fold lower than $G_{α}$ and $G_{α}$, respectively, in rat brain cortex (25). The relative abundance in bovine brain is $G_{α}$ > $G_{β_{α}}$ = 10; $G_{β_{α}}$ could not be detected because of low affinity of the antisem for bovine $G_{β_{α}}$ (26). The relative amounts of $G_{α}$, subunits eluted by affinity chromatography with immobilized $G_{β_{α}}$ subunits are similar with either rat or bovine brain membranes as the source (20). These data suggest that the relative abundance of G proteins in bovine brain is $G_{β_{α}} > G_{β_{α}} > G_{β_{α}}$.

There may be other reasons for the low levels of $G_{β_{α}}$ in affinity column eluates. It is possible that coupling is disturbed during solubilization and purification of receptor-G protein complexes and that coupling is regulated by unknown factors. If solubilized complexes of $A_1$ receptors and $G_{α}$ are not as stable as complexes of receptors and $G_{α}$ or $G_{α}$, then receptor-G, complexes may dissociate prior to or during affinity chromatography. It is also conceivable that receptor-G, complexes bind but do not elute from the affinity column with GTP or NEM. However, the following observations support the notion that all $A_1$ receptor-G protein complexes that adhere to the column are eluted in response to GTP or NEM. (i) Although $A_1$ receptor binding to various fractions accounted for only 20$\%$ of the total receptors that adhered to the column, the recovery of $[^{35}]$GTPyS binding sites was
close to the number of receptors that adhered to the affinity column (17). This suggests that all G proteins that are bound as receptor-G protein complexes are eluted from the affinity column by GTP or NEM, but some receptors are not eluted or lose the capacity to bind radioligands. (ii) The elution profile of [35S]GTPγS binding revealed that G proteins specifically bound to the column as receptor-G protein complexes were rapidly eluted by GTP, suggesting that quantitative differences were not the result of variable kinetics of release from the affinity column (27). (iii) The specificity of the affinity column for G proteins complexed with adenosine receptors was confirmed by demonstrating that depletion of solubilized receptors by affinity chromatography over an antagonist column (28) or competitive blockade of receptors with 10 μM R-PIA (17) abolished retention of G proteins to the agonist affinity column. (iv) When the affinity column was eluted with 0.2% SDS after GTP/NEM elution, the SDS eluate did not contain immunoassayable Gα2 or Gα1 (data not shown). This rules out the possibility that Gα2 was found in low abundance because it cannot be eluted from the affinity column.

A1 receptors may couple to multiple G proteins only after they are solubilized. However, it has been shown that GTP has differential effects on agonist radioligand binding to subpopulations of receptors in porcine atrial membranes (29). This has been taken as evidence that A1 receptors are coupled to multiple G proteins in membranes as well as after solubilization in detergents. The data from these reconstitution studies support the notion that A1 receptors can interact with multiple G proteins in the absence of detergent (Fig. 2). The excess of G proteins over receptors required for optimal reconstitution is similar to the excess required for reconstitution of agonist binding activity of muscarinic (22) and μ-opioid receptors (16).

Prolonged incubation of primary cultures of rat adipocytes with R-PIA has been reported to cause down-regulation of several different pertussis toxin-sensitive G proteins. Longabaugh et al. (30) showed that treatment with R-PIA down-regulated approximately 60% of both Gα1 and Gα2 with no change in the levels of Gβ1 or Gα1. Green et al. (31) found that 100% of Gα1 and Gα2 and only 50% of Gα3 were down-regulated. Down-regulation of Gα1 suggests but does not prove that A1 adenosine receptors can couple to multiple G proteins. Our data provide additional evidence for coupling of brain A1 receptors to at least two out of the three G proteins (G1 and G2) and also to Gα (Fig. 2), which is not present in adipocytes (31). Gα in the brain appears to be involved in the coupling of receptors to the activation of K+ channels (10) and the inhibition of Ca2+ channels (26, 32). Activation of K+ channels is one of several different mechanisms by which A1 receptors inhibit the release of excitatory neurotransmitters in brain (33).

These data are consistent with the idea that a single adenosine receptor polypeptide can interact with multiple G proteins. However, it is possible that affinity chromatography results in the purification of a mixture of subtly different A1 receptor subtypes. Such subtypes have been proposed on the basis of differences in structure activity relationships for various A1 receptor-mediated responses (34). Hence we cannot exclude the possibility that the various G proteins that we detect are in fact coupled to multiple A1 receptor subtypes. On the other hand, purified adenosine receptors appear to consist of a single 35–36-kDa glycoprotein, and the binding of several ligands is consistent with the existence of a homogeneous population of binding sites capable of coupling to multiple G proteins (17). Also, the reconstitution of agonist binding to A1 receptors by G proteins is not additive inasmuch as the amount of [35S]ABA binding is similar when two G proteins (G1 + G2, G1 + G3, G2 + G3) are used together for reconstitution each at a concentration required for maximal restoration of high affinity binding activity. This suggests that purified A1 receptor preparations comprise a single population of A1 receptors which can couple to multiple G proteins.

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REFERENCES

2 R. Munshi and J. Linden, unpublished data.